JOURNAL OF CLINICAL MICROBIOLOGY, June 1994, p. 1492–1496
0095-1137/94/$04.00+0

Vol. 32, No. 6

Detection of *Bartonella (Rochalimaea) quintana* by Routine Acridine Orange Staining of Broth Blood Cultures

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Received 4 October 1993/Returned for modification 15 December 1993/Accepted 14 February 1994

***Bartonella quintana* was isolated from 34 BACTEC nonradiometric aerobic resin blood cultures for 10 adults. Nine patients were initially diagnosed by routine acridine orange staining of routine cultures that had been incubated for 8 days. All subcultures grew on chocolate agar within 3 to 12 days (median, 6 days). The PLUS 26 high-volume aerobic resin medium, combined with acridine orange stain and subculture, is an effective system for detection and isolation of *B. quintana* from blood.**

The recovery of *Bartonella* spp. from clinical specimens has become increasingly important. *Bartonella quintana*, long known as the agent of trench fever, a febrile syndrome rarely recognized in nonepidemic situations, recently has been reported as a likely cause of bacillary angiomatosis (6) and endocarditis (18). The newly described *Bartonella elizabethae* also has been isolated from a patient with endocarditis (2). These findings and the recent description of *Bartonella henselae* and its association with cat scratch disease, bacillary angiomatosis, peliosis hepatis, and bacteremia (3, 6, 8, 12, 14, 20–22) have prompted increased efforts to recover these organisms from clinical specimens. Detection of these fastidious, slowly growing organisms is essential for proper management of both immunocompetent and immunocompromised patients.

Bartonella spp. have been isolated from blood specimens by several different methods, including the Septi-Chek biphasic system (8), subculture of BACTEC 460 bottles (2, 8), the BACTEC NR-660 infrared CO_2 detection system (18), direct plating of whole blood onto agar (12, 19), and the Isolator lysis-centrifugation system (6, 8, 17). The speed and frequency of isolation of *Bartonella* spp. from lysis-centrifugation cultures have led several authors to recommend this method for blood culturing (8, 15, 17, 21). However, because the lysis-centrifugation system remains prone to contamination and requires extensive manipulation, including centrifugation, many laboratories have not adopted this method. There is a need to improve detection and isolation of *Bartonella* spp. from currently available broth blood culture systems.

Our laboratory uses the BACTEC NR-660 system for blood cultures. Because this system may not detect organisms that produce little CO_2, we stain an aliquot from each aerobic bottle with the acridine orange (AO) stain on day 8 and subculture those bottles with positive AO stains. The AO stain has been reported as an acceptable alternative to subculture of blood culture bottles (4, 5, 10, 13) and is known to be more sensitive than the Gram stain (7, 11).

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Here we report the isolation of *B. quintana* from 34 BACTEC aerobic resin bottles. Prompt detection of most pretreatment isolates was accomplished by routine AO staining, and all isolates were recovered by extended incubation of sheep chocolate agar subcultures.

It should be noted that all *Rochalimaea* species recently have been transferred to the genus *Bartonella* (1).

MATERIALS AND METHODS

Sources of strains. *B. quintana* ATCC VR 358 and ATCC VR 960A and *B. elizabethae* ATCC 49927A were obtained from the American Type Culture Collection. *B. henselae* ATCC 49793 and ATCC 49882 were provided by one of us (D. F. Welch). Clinical isolates were recovered from patients at Harborview Medical Center in the first 6 months of 1993.

Cultures. A routine adult blood culture consisted of one high-volume aerobic resin PLUS 26 bottle and one standard anaerobic NR 7A bottle (Becton Dickinson Diagnostic Instrument Systems, Sparks, Md.) incubated at 35°C. Aerobic bottles were continuously rotated during the first 24 h of incubation. All bottles were monitored by the BACTEC NR-660 infrared CO_2 detection system for the first 5 days and then incubated without monitoring for 2 additional days. Before discard of negative cultures on the eighth day, each anaerobic bottle was again tested for CO_2 production, while an aliquot from each aerobic bottle received an AO stain as described below. If bacteria were seen on the AO stain, a subculture was made onto chocolate agar, heart infusion blood agar, and brucella blood agar. The heart infusion blood and chocolate agars were incubated in an aerobic atmosphere in increased CO_2 for at least 2 days. The brucella blood agar was incubated in an anaerobic GasPak jar (BBL Microbiology Systems, Cockeysville, Md.) for 4 days. Each agar was prepared with 5% sheep blood, and the chocolate agar, containing GC Medium Base (Difco Laboratories, Detroit, Mich.), was supplemented with 1% IsoVitaleX (Becton Dickinson Microbiology Systems, Cockeysville, Md.).

When organisms with *Bartonella* morphology were seen upon AO staining, the subculture on chocolate agar was incubated until growth was visible. On the basis of experience with the first few blood cultures with *B. quintana*, later blood

cultures with AO stains suggesting *Bartonella* spp. were subcultured onto only chocolate agar.

For selected specimens, the ISOSTAT Microbial System (Wampole Laboratories, Cranbury, N.J.) was used according to the manufacturer's instructions. The lysed concentrate from an Isolator 10 tube was cultured in equal amounts on four media. The brucella blood agar was incubated anaerobically, whereas chocolate, heart infusion blood, and buffered charcoal yeast extract agars were incubated aerobically in increased CO_2 in polyethylene bags for at least 21 days at 35°C.

Agar media were prepared by the University of Washington Medical Center Clinical Microbiology Laboratory, Seattle, and inoculated within 2 weeks of the preparation date. All aerobic incubation of agar media occurred at 35°C in an incubator which maintained 5 to 8% CO_2 and 85 to 94% relative humidity levels.

Stains. A smear for the AO stain was made with a drop of blood culture broth, dried at room temperature, rinsed with methanol, and again dried in air. AO stain (Prepared Media Laboratory, Inc., Tualatin, Oreg.) was applied for 2 min, and the slide was rinsed with tap water and air dried. Smears were scanned with a Zeiss standard microscope with a 100-W halogen light source, epi-illumination, and a BP 450-490–FT 510–LP 520 blue excitation filter set.

The Enhanced Gram stain (Carr Scarborough Microbiologicals, Inc., Stone Mountain, Ga.) was performed on broth cultures with presumptively positive AO stains. The unique component of this modified Gram stain is the Gram Enhancer solution, containing fast green and tartrazine dyes which impart a gray-to-green color to debris but do not affect the Gram reaction of organisms. Feathered-edge smears were prepared, dried in air, rinsed with methanol, and again air dried. Stains were applied according to the manufacturer's instructions, with the exception that the Gram Safranin stain time was extended to 5 min. The counterstain for routine Gram stains was 1.0% aqueous safranin, which occasionally was replaced by a 1/20 dilution of Kinyoun carbolfuchsin.

Identification methods. Each isolate was tested for preformed enzyme activity by using the MicroScan Rapid Anaerobe Identification Panel (Baxter Diagnostics, Inc., Deerfield, Ill.). The inoculum consisted of growth from 3- to 5-day-old cultures on chocolate agar. Panel inoculation, incubation, and interpretation were done as recommended by the manufacturer for anaerobic organisms, including an inoculum density approximating that of a 5.0 McFarland turbidity standard. Reactions were recorded within 3 min after reagent addition. Oxidase and catalase tests were done with 1.0% *N,N*-dimethyl-*p*-phenylenediamine dihydrochloride (Eastman Kodak Co., Rochester, N.Y.) and 3% H_2O_2 reagents, respectively. Urease testing was performed by heavily inoculating a Christensen's urea slant, which was incubated for 5 days at 35°C.

Cellular fatty acid (CFA) analysis was performed by gas-liquid chromatography with the Microbial Identification System (MIDI, Newark, Del.). The Microbial Identification System included a model 5890A gas chromatograph with capillary column, flame ionization detector, automatic sampler, integrator, and microcomputer (Hewlett-Packard, Palo Alto, Calif.). A standard fatty acid mixture was purchased from MIDI. The manufacturer's protocol was followed for all stages of saponification, methylation, extraction, and chromatography procedures, except for culturing of cells on sheep chocolate agar in a CO_2-enriched atmosphere. Peaks were automatically integrated and identified, including percent composition.

Isolates were identified by an immunofluorescence-antibody method employing mouse antisera which react specifically with *B. quintana* and *B. henselae* as previously described (16, 20). Bacteria were harvested from agar cultures, formalin fixed, washed in phosphate-buffered saline (PBS), and adjusted to a density of approximately 10^8 CFU/ml. One-microliter aliquots were air dried in wells of glass slides. Antisera were added, and the mixtures were incubated for 30 min at 37°C in a moist chamber. After two PBS washes, goat anti-mouse immunoglobulin G-fluorescein isothiocyanate conjugate (Chemicon, Temecula, Calif.) was added for 30 min of incubation at 37°C. Following additional rinses and air drying, the slides were prepared with buffered glycerol mounting medium and coverslips for examination by epifluorescence microscopy using an Olympus BH-2 microscope and a mercury light source.

DNA analyses for identification of *Bartonella* species were done by the methods of Matar et al., whose methods for cell lysis, PCR amplification, and restriction enzyme digestion of amplicons were previously described (9). Briefly, a PCR-amplified DNA fragment which included the spacer region between the 16S and 23S rRNA genes and a portion of the gene coding for 23S rRNA was digested with the restriction endonucleases *Hae*III and *Alu*I. The resulting restriction fragment patterns were examined for patterns characteristic of each *Bartonella* species (9).

RESULTS

The 34 blood cultures yielding *B. quintana* were processed with approximately 6,000 other BACTEC blood culture sets between January and June 1993. These positive cultures were from specimens drawn from 10 adult patients to be described in a separate paper (17a).

On AO-stained smears, *B. quintana* organisms appeared as pale orange amorphous clumps that contrasted with the green color of deteriorated blood cells (Fig. 1). Upon thorough examination, tiny bacilli sometimes could be seen separately or along the edge of large clumps. Follow-up routine Gram stains were negative, even with carbolfuchsin counterstaining. However, clumps of bacteria could be seen on an Enhanced Gram stain in areas of the slide which had little background debris and good separation of the gray-to-green erythrocytes (Fig. 1, bottom panel). With the Enhanced Gram stain, we discovered that *Bartonella* organisms were more intensely pink when the safranin stain time was increased to 5 min. Attempts to substitute carbolfuchsin for safranin in the Enhanced Gram stain failed because the nonbacterial debris took on a fuchsia color.

In 14 of 17 positive aerobic resin bottles (82%) from nine patients who had not received antibiotic therapy, *B. quintana* organisms were detected by routine AO screening on day 8 (Table 1). One of the AO stains included as a routine AO-positive stain was inadvertently performed on day 6. One additional positive culture was detected by a repeat AO stain on day 12, and two cultures were detected by blind subculture. Two pretherapy cultures of blood drawn the day before the patient had two positive cultures were negative by routine AO and never subcultured (data not shown). Two additional pretherapy cultures for a different patient grew coagulase-negative staphylococci, and neither AO staining nor extended incubation was performed to rule out *Bartonella* spp.

In general, the AO stain was less useful after patients had received antibiotics (Table 2). Of 17 positive cultures of blood drawn after therapy, 7 (41%) were detected by routine AO, 2 by repeat AO, and 8 by blind subculture. Blind subcultures and repeat stains were not performed on a regular schedule but were done because the patients were known or suspected to be infected with *Bartonella* spp.

The BACTEC NR-660 system did not detect CO_2 produc-

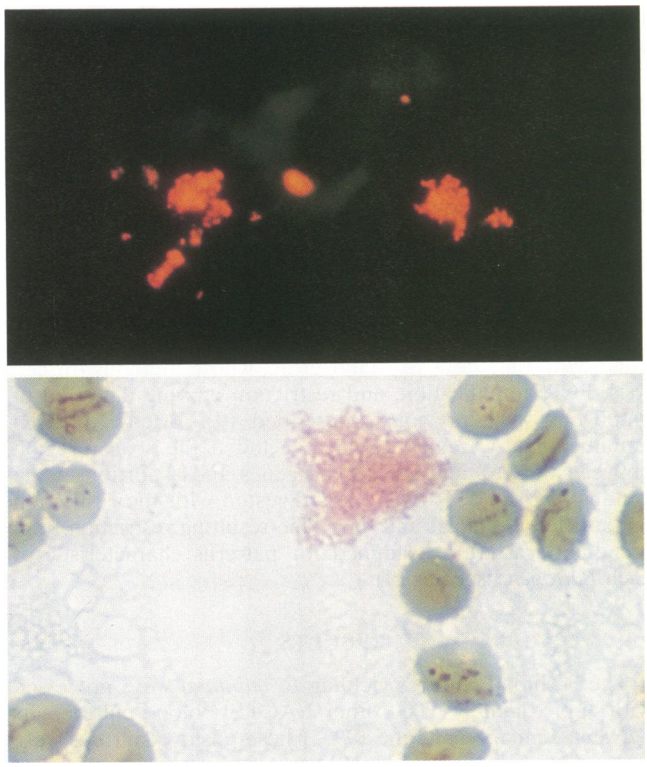

FIG. 1. (Top panel) AO stain of blood culture broth, with orange clumps of *B. quintana* and green debris from blood cells. Magnification, ×1,000. (Bottom panel) Enhanced Gram stain of blood culture broth demonstrating a clump of *B. quintana* cells and gray-green erythrocytes. Magnification, ×1,000.

tion within the first 5 days of incubation in any of the 34 blood culture sets containing *B. quintana*. All positive PLUS 26 bottles had growth values (GVs) of <30 on day 1 and <35 on days 2 through 5. After day 5, CO_2 monitoring of PLUS 26 bottles was performed irregularly. All of the AO-positive bottles tested on day 8 (n = 8) had GVs of <20. Of the 11 positive bottles incubated and sporadically tested beyond day 8, 7 continued to have low GVs, whereas 4 had GVs between 35 and 47 recorded at 22, 30, or 36 days after phlebotomy.

Although each blood culture set included both an aerobic PLUS 26 and an anaerobic NR 7A bottle, only the aerobic bottles grew *B. quintana*. Most anaerobic bottles were screened only with the routine protocol. The highest GV achieved by a companion NR 7A bottle in the first 5 days of incubation was 35. Chocolate agar subcultures made for this and six other anaerobic bottles with positive companion aerobic bottles were negative.

Subcultures performed after positive routine AO screening grew within 3 to 8 days, with antibiotic therapy having little effect. The median times to growth for subcultures of both pre- and posttherapy bottles with positive routine AO stains were 6 and 5 days, respectively, with a combined range for isolation times of 11 to 16 days. Most positive blind subcultures grew within the range of time described for subcultures of bottles with positive AO smears; however, two blind subcultures required 12 days of incubation. These two subcultures were for the same patient, who had no positive AO stains. The total time to isolation varied with the timing of subcultures.

In three instances, Isolator system and BACTEC cultures were inoculated simultaneously (Table 3). Organisms were isolated from all three PLUS 26 bottles, but one of the three Isolator cultures failed to grow. In the two positive pairs, the detection of organisms was 1 and 4 days earlier for PLUS 26 bottles, although the time to isolation was shorter for Isolator cultures. Colonies on Isolator cultures were too numerous to count, and for one culture colonies appeared 7 days earlier on sheep heart infusion agar than on chocolate agar. The two patients who had positive Isolator cultures had received antibiotics 2 to 3 weeks previously, whereas the negative culture was for a third patient with no history of antibiotic therapy.

B. quintana growth first appeared as tiny, translucent to light tan punctate colonies that were somewhat gummy and adherent. Colonies that required 12 days to grow were notably more adherent. Stains revealed small, faintly staining gram-negative rods that often were slightly curved, as pictured previously (12, 17). The bacilli tended to form clumps that were more easily seen when the conventional Gram stain included the carbolfuchsin counterstain.

All isolates were oxidase, catalase, and urease negative. The definitive identifications of isolates from the 10 patients were based on reactivity with antiserum specific for *B. quintana* and

TABLE 1. *B. quintana* detection and isolation from positive PLUS 26 blood cultures for nine untreated patients

Result of AO stain		No. of cultures (n = 17)	Days to positive AO stain	Median days (range) to growth from date of:	
Routine (day 8)	Repeat			Subculture	Blood draw
+[a]	ND[b]	14	8	6 (4–8)	14 (12–16)
−	+	1	12	3	15
−	ND	2		10 (7–12)	22 (16–26)

[a] One PLUS 26 bottle stained on day 6; one bottle was PEDS PLUS medium.
[b] ND, not determined.

TABLE 2. *B. quintana* detection and isolation from positive PLUS 26 blood cultures for five treated patients

Result of AO stain		No. of cultures (n = 17)	Days to positive AO stain	Median days (range) to growth from date of:	
Routine (day 8)	Repeat			Subculture	Blood draw
+	ND[a]	7	8	5 (3–6)	13 (11–14)
−	+	2	30	8 (5–10)	38 (35–40)
−	−[b] or ND	8		6 (3–12)	22 (18–32)

[a] ND, not determined.
[b] Seven bottles had negative repeat stains on the subculture date.

TABLE 3. Days to detection of *B. quintana* from paired PLUS 26 and Isolator cultures for three patients

Culture pair	Days to result by BACTEC		Days to agar growth by Isolator	Isolator colony count
	Positive AO stain	Agar growth		
1	8	13	12	TNTC[a]
2	8	14	9[b]	TNTC
3	8	12	NG[c]	

[a] TNTC, too numerous to count.
[b] Colonies visible on blood agar on day 9; growth on chocolate agar appeared on day 16.
[c] NG, no growth.

TABLE 4. CFAs of patient isolates and reference strains of *Bartonella* spp.

Strain	Major fatty acids (% of total)[a]			
	16:0	17:0	18:1[b]	18:0
B. henselae				
ATCC 49793	19		57	22
ATCC 49882	17		60	21
B. quintana				
ATCC VR 358	12	2	67	18
ATCC VR 960A	15	3	66	16
B. elizabethae ATCC 49927A[c]	18	25	39	9
Patient isolates (n = 10)[d]	16–19	2–3	60–65	13–18

[a] CFAs of ≤1% are not listed.
[b] Represents 18:1 w7c/w9t/w12t (not separable).
[c] Additional fatty acids were 15:0 (3%) and 17:1w6c (6%).
[d] One representative isolate from each patient.

a lack of reactivity with anti-*B. henselae* serum. Each isolate also had restriction fragment patterns from amplicon digests that were characteristic of *B. quintana* (9). The biotype number in the MicroScan Rapid Anaerobe Identification Panel for representative isolates from the 10 patients was 10077640. Reference strains of *B. henselae* and *B. quintana* also yielded profile number 10077640, which differs by one reaction from 10073640, the previously published profile number for *B. quintana* (20). The type strain of *B. elizabethae* yielded a unique profile number (10077240) in the MicroScan panel.

Results from fatty acid analyses of reference strains and clinical isolates from the 10 patients are presented in Table 4. The three major fatty acids for all *Bartonella* spp. except *B. elizabethae* were $C_{18:1}$, $C_{18:0}$, and $C_{16:0}$.

DISCUSSION

Several methods have been used to isolate *Bartonella* spp. from blood specimens, but the Isolator tube system has been the most successful commercial system to date. Both *B. quintana* and *B. henselae* have been recovered with the Isolator system (6, 8, 12, 20, 21). One *B. quintana* and eight *B. henselae* isolates were recovered from Isolator cultures but not from concurrent 7-day Septi-Chek cultures (21). The times to detection of macroscopic growth of *B. henselae* have been reported as ranging from 5 to 15 (mean = 9) days with the Isolator system (21) and from 40 to 49 days with the Septi-Chek system (8).

Either the BACTEC radiometric and infrared CO_2 detection systems have not detected any CO_2 produced by *Bartonella* spp. or the CO_2 detection was significantly delayed. In one report, Gram stains of 14 radiometric BACTEC bottle cultures with negative GVs revealed gram-negative rods after 7 to 10 days of incubation, but *B. henselae* was isolated from only one bottle (8). *B. elizabethae* was isolated from 3-week-old radiometric BACTEC bottle cultures which had negative GVs (2). *B. quintana* was detected by positive GVs in two BACTEC PLUS 26 bottles but only after 28 and 42 days of incubation (18).

Our results also indicate that the BACTEC infrared CO_2 detection system cannot be depended upon as the sole method of detection of *B. quintana*. None of our cultures produced sufficient CO_2 to indicate growth within the first 5 days of incubation, and the few that exceeded threshold CO_2 levels did so only after 3 weeks of incubation. However, we found that *B. quintana* organisms were able to grow well in BACTEC aerobic resin media and that they could be detected easily by using more sensitive stain techniques and extended incubation of sheep chocolate agar subcultures.

The typical clumps of *B. quintana* are readily overlooked on a routine Gram-stained smear because both blood cell debris and organisms appear as gram-negative aggregates, whereas with the AO and Enhanced Gram stains debris and organisms appear in contrasting colors. It should be noted that *Mycoplasma* spp. can produce clumps similar to those of *Bartonella* spp., but in our experience, the *Mycoplasma* staining is denser and more brilliant with both the Enhanced Gram and AO stains, and individual organisms are not seen.

In a limited comparison, involving only three paired Isolator and PLUS 26 cultures, the AO stain detected *B. quintana* in PLUS 26 bottles before the Isolator growth appeared, although colonies were available earlier with the Isolator cultures. It is also noteworthy that the Isolator system failed to detect growth from the blood of one patient, who was not on antibiotics, while the companion PLUS 26 bottle was positive. Furthermore, our day 8 detection of 21 positive cultures by AO stain compares well with the average 9-day detection time for lysis-centrifugation cultures reported elsewhere (21). Our positive stain from a 6-day blood culture suggests that the AO stain can detect *B. quintana* earlier than day 8. Further comparative studies, including patients infected with other *Bartonella* species, are needed to determine the relative sensitivities of these two blood culture systems.

AO stains were less likely to be positive for cultures of blood drawn after patients had received antibiotic therapy. The routine AO stain also was negative for a pretherapy culture, later found to be positive on a blind subculture, from the blood of a patient with suspected endocarditis. Because of the possibility of fluctuating numbers of organisms in the blood and differences in bacterial strains, the AO stain must be accompanied by a subculture held for at least 13 days.

CFA profiles demonstrated percentages consistent with profiles of *Bartonella* spp. The amount of $C_{18:0}$ from clinical isolates and reference strains of *B. quintana* was ≤18%, whereas the reference strains of *B. henselae* had greater amounts ranging from 21 to 22%, which is consistent with earlier work (21). However, differentiation of these two species based on CFA profiles is not reliable. Various authors have reported the $C_{18:0}$ contents of *B. quintana* and *B. henselae* to range from 16 to 23% and 18 to 30%, respectively (2, 3, 8, 17, 18, 21). Furthermore, it has been shown that the medium source or conditions can markedly influence the fatty acid analyses for these species (8). We found that the age of the culture influences the fatty acid composition of *Bartonella* spp. (data not shown). The suggestion that $C_{16:0}$-to-$C_{18:0}$ ratios can distinguish these two species (17) has not been consistently confirmed.

We obtained the same MicroScan Rapid Anaerobe Identification Panel profile number for *B. quintana* and *B. henselae* isolates. An inoculum equivalent to a 5.0 McFarland standard consistently yielded the profile number 10077640, which differs slightly from results in a previous report (20). Further testing revealed that biochemical reactions were dependent on inoculum density and to some extent on the growth medium (data not shown). Until definitive studies of the effects of these variables have been completed, we recommend fluorescent-antibody testing or DNA analysis to differentiate these two species.

Detection and isolation of slowly growing, fastidious organisms such as *Bartonella* spp. in conventional broth blood cultures is difficult because these organisms produce little or no CO_2 or visible growth. Our results for patients who had not received antibiotic therapy indicate that an AO stain followed

by subculture onto chocolate agar in increased humidity and CO_2 for 2 weeks of incubation should provide a relatively rapid and sensitive method for detection and isolation of *B. quintana* from BACTEC aerobic resin blood cultures.

ACKNOWLEDGMENTS

This report would not have been possible without major efforts by the technologists from Harborview Medical Center and University of Oklahoma Health Sciences Center. We gratefully acknowledge L. N. Slater for generously providing the antisera to *B. quintana* and *B. henselae*.

Carr Scarborough Microbiologicals, Inc., generously provided the funds to print Fig. 1 in color.

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